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# **Assay and inhibition of the purified catalytic domain of diacylglycerol lipase beta**

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**Title**

Assay and inhibition of the purified catalytic domain of diacylglycerol lipase beta

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## Abbreviations and Textual Footnotes

2-AG - 2-arachidonoylglycerol; AA – Arachidonic acid; CB – cannabinoid; DAGL – Diacylglycerol Lipase; DiFMUO - 6, 8-difluoro-4-methylumbelliferyl-octanoate; eCB – endocannabinoid; GST - glutathione S-transferase; GST-DAGL $\beta$  CD - glutathione S-transferase diacylglycerol lipase  $\beta$  catalytic domain; PNPB - p-nitrophenyl butyrate; Sf9 - *Spodoptera frugiperda*; THL - tetrahydrolipstatin

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**Abstract**

The diacylglycerol lipases (DAGL $\alpha$  and DAGL $\beta$ ) hydrolyse DAG to generate 2-arachidonoylglycerol (2-AG), the principal endocannabinoid and main precursor of arachidonic acid (AA). The DAGLs make distinct tissue specific contributions towards 2-AG and AA levels and therefore selective modulators for these enzymes could play crucial roles towards harnessing their therapeutic potential. Relatively high-throughput assays have recently been reported for DAGL $\alpha$  and have proven useful towards the characterization of inhibitors of this enzyme. Similar assays are also warranted for DAGL $\beta$  which was the aim of this study. We first adapted previously reported DAGL $\alpha$  membrane assays (using PNPB and DiFMUO as substrates) to measure recombinant DAGL $\beta$  activity in membranes. In contrast to results with DAGL $\alpha$ , both substrates provided a relatively limited signal window for measuring DAGL $\beta$  activity, however, an improved window was obtained when employing a third commercially available substrate, EnzChek. In order to further improve on the assay parameters, we successfully purified the glutathione S-transferase (GST) tagged catalytic domain of DAGL $\beta$ . Activity of the enzyme was confirmed using EnzChek as well as two DAGL inhibitors (THL and OMDM-188). The purified DAGL $\beta$  catalytic domain assay described here provides the basis for a relatively clean and convenient assay with the potential to be adapted for high-throughput drug discovery efforts.

## Introduction

The diacylglycerol lipases (DAGL $\alpha$  and DAGL $\beta$ ) generate a signalling pool of 2-arachidonoylglycerol (2-AG) for endocannabinoid (eCB) signalling via the CB1 and/or CB2 receptors which serves numerous functions during development and in the adult. For example, DAGL-dependent eCB signalling regulates axonal growth and guidance during development<sup>1-6</sup>. In the adult two forms of plasticity are controlled by DAGL-dependent eCB signalling and these are synaptic plasticity in the form of retrograde synaptic transmission throughout the brain<sup>7,8</sup> and cellular plasticity in the form of adult neurogenesis in the hippocampus and sub-ventricular zone<sup>1,7,9,10</sup>. The DAGLs have a second important function, and this is the generation of 2-AG as a substrate for monoacylglycerol lipase (MAGL) to maintain arachidonic acid (AA) levels in various tissues including the brain, liver and adipose<sup>7,11,12</sup>.

The DAGLs are emerging as interesting therapeutic targets<sup>13-15</sup>. In this context, DAGL activity drives eCB signalling and drugs that modulate other steps in this pathway have shown beneficial effects in obesity<sup>16-19</sup> and pain<sup>20,21</sup> amongst many other diseases<sup>22</sup>. More recently, the DAGL-MAGL pathway has been implicated to operate independently of the eCB pathway in inflammatory responses associated with Alzheimer's disease<sup>23</sup>, Parkinson's disease<sup>24</sup> and peripheral inflammation<sup>13,24</sup>. It is noteworthy that DAGL $\alpha$  and DAGL $\beta$  make distinct tissue specific contributions towards 2-AG and AA levels and therefore selective modulators for these enzymes are likely to be essential to harness their therapeutic potential from both a safety and efficacy perspective<sup>7,25</sup>. For instance, recent studies have highlighted DAGL $\beta$  as a target for inflammatory diseases via mechanisms independent of the cannabinoid receptors whereas DAGL $\alpha$  is responsible for 2-AG mediated CB1 signalling at the synapse and therefore non-selectively targeting DAGL $\beta$  (over DAGL $\alpha$ ) could risk

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3 disrupting CB1 signalling at the synapse<sup>13,23–25</sup>. It is therefore crucial to factor in selectivity  
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5 assays to address this in drug discovery programmes.  
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8 Over the years, a wide range of assays have been developed to measure DAGL activity which  
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10 have been utilized for the characterization, discovery and/or development of established as  
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12 well as novel DAGL inhibitors. Mass spectrometry based assays that utilise synthetic DAG as  
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14 the native substrate and the measurement of 2-AG as the product have been reported<sup>26,27</sup>. A  
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16 thin layered chromatography based radio-assay involving radio-labelled DAG has also been  
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18 utilized to measure DAGL activity<sup>2,28</sup>. Several fluorescently labelled activity based probes  
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20 with applications in gel based assays have been developed for the DAGLs, ranging from the  
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22 generic serine hydrolase binding probe – fluorophosphonate<sup>27</sup> to more recent probes with  
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24 ever improving selectivity for DAGL $\alpha$  and DAGL $\beta$ <sup>13,29,30</sup>. Recently, higher throughput  
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26 assays suitable for drug-discovery programs for the DAGLs have been reported. Johnston et  
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28 al reported on a fluorescence resonance energy transfer assay using ether lipid reporters and  
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30 which is of particular utility when using DAGLs purified to homogeneity (due to hydrolysis  
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32 by non DAGL enzymes in crude samples)<sup>28</sup>. Van der Wel et al reported on a DAGL $\alpha$  native  
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34 substrate assay using a coupled enzyme approach adapted from a previously reported MAGL  
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36 assay<sup>31,32</sup>. Pedicord et al., reported on simple economical recombinant DAGL $\alpha$  membrane  
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38 based assays utilizing the fluorogenic 6, 8-difluoro-4-methylumbelliferyl-octanoate  
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40 (DiFMUO) and the chromogenic p-nitrophenyl butyrate (PNPB) as surrogate substrates<sup>33,34</sup>.  
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47 Similar assays are also warranted for DAGL $\beta$  and so in the present study, we focussed on the  
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49 development of DAGL $\beta$  activity assays to further complement the currently available panel  
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51 of DAGL assays for drug discovery programmes. We first generated and characterized a  
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53 clonal line of human U2OS osteosarcoma cells transfected with a construct expressing the  
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55 epitope (V5) tagged human DAGL $\beta$  transgene for expression of the recombinant enzyme.  
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57 We attempted to adapt previously reported DAGL $\alpha$  membrane assays (using PNPB and  
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DiFMUO as substrates) to measure DAGL $\beta$  activity using membranes prepared from these cells but with limited success due to relatively modest signal windows. However, a much improved window was obtained using a third commercially available substrate, EnzChek. In order to further improve on the assay parameters, we successfully purified the N-terminally glutathione S-transferase (GST) tagged catalytic domain of DAGL $\beta$ , which in conjunction with EnzChek, provided the basis for a relatively clean, convenient, and high-throughput assay and thereby an attractive tool for drug discovery and development efforts.



**Experimental Procedures (Materials and Methods)**

**Reagents**

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

**Generation of cell line stably expressing DAGL $\alpha$ / $\beta$  with a C-terminal V5 tag**

Full length human DAGL $\alpha$ / $\beta$  was cloned into the pcDNA6.2/V5-DEST vector (Invitrogen) to enable expression of the transgene with a C-terminal V5 tag. These constructs were individually transfected (Lipofectamine 2000, Invitrogen) into a human U2OS osteosarcoma cell line that for the purpose of future studies was also genetically modified to express a CB1 receptor reporter construct (Tango™ CNR1-bla U2OS cells from Invitrogen) and stable transfected clones were selected using blasticidin (4 $\mu$ g/ml). The cell lines stably expressing DAGL $\alpha$ -V5 (V5 $\alpha$ 11) or DAGL $\beta$ -V5 (V5 $\beta$ 4) were subsequently established following verification by Western blotting and immunocytochemical analyses using a V5 antibody (mouse, Invitrogen).

**Membrane preparation**

Membranes were prepared following a previously published method<sup>33</sup>. Briefly, cells cultured in 10cm dishes (~90% confluent) were scraped in 1ml (per dish) of lysis buffer (20mM HEPES pH7.0, 2mM DTT, 0.25M sucrose, 10mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub> and 1x Roche 'Complete' protease inhibitor) and then homogenised on ice using a Polytron (PT 1200 E) homogeniser (three 7 seconds bursts at the maximum setting with 30 seconds interval in between). The homogenates were then subjected to centrifugation at 100,000g for 30 minutes

at 4°C. The supernatants were discarded and the pellets (membrane preparations) were resuspended in 200µl of sucrose free lysis buffer, using the homogeniser. Membrane aliquots were stored at -80°C.

### Immunocytochemistry

Cells (10,000/well) were seeded onto poly-lysine coated coverslips and cultured overnight after which they were fixed in 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were washed three times with phosphate buffered saline (PBS) and then permeabilised with 0.2% Triton X-100-PBS for 10 minutes. The permeabilised cells were washed three times with PBS, blocked with 1% BSA-PBS (block solution) for 30 minutes and then incubated with a V5 antibody (mouse, Invitrogen, diluted 1/1000 in block solution) for 1 hour at room temperature. The cells were then washed with PBS and incubated for a further hour with an AlexaFluor 488 secondary antibody (mouse, Invitrogen, diluted 1/2000 in block solution) and the nuclear stain Hoechst 33258 (Invitrogen, diluted 1/10,000 in block solution). After three washes with PBS, the coverslips were mounted onto microscope slides and images of the immunostained cells were collected using the Carl Zeiss LSM 710 microscope and the Carl Zeiss Zen software (version 1.0.1.0).

### Expression and purification of DAGLβ catalytic domain

The human DAGLβ catalytic domain sequence corresponding to amino acids 228-672 was cloned into the pDEST20 vector (Invitrogen) to enable expression of the DAGLβ catalytic domain with an N-terminus GST tag (GST-DAGLβ CD) using the baculovirus expression system. For the baculovirus generation and expression Sf9 (*Spodoptera frugiperda*) insect

cells along with SF-900 II SFM media (Invitrogen) were used. Recombinant baculoviruses (P1) were generated and amplified using the Sf9 cells and the GST-DAGL $\beta$  CD pENTR construct following the Bac-to-Bac system (Invitrogen). In order to amplify the virus, 200mls Sf9 cells at a density of  $1.2 \times 10^6$  cells/ml in 1L Erlenmeyer flasks were infected with 500 $\mu$ l of P1/2 virus and incubated in a shaker at 27.5°C (110rpm) for 4-5 days (or until viability <80%). The P2/3 virus (supernatant) was then collected by centrifugation (3,000g for 10 minutes). The viruses were stored in the dark at 4°C in the presence of 5% foetal calf serum. For expression, 10ml P2/3 virus was added to 200ml Sf9 cells at a density of  $2 \times 10^6$  cells/ml (in 1L Erlenmeyer flask). The cells were cultured for 48 hours (27.5°C and 110rpm) and then harvested by centrifugation at 3000g. Cell count at the time of harvest was generally  $\sim 3-4 \times 10^6$ /ml ( $\sim 75\%$  viable).

**Purification of the GST tagged DAGL $\beta$  catalytic domains**

*Cell lysis:*  $3 \times 10^9$  cells were resuspended ( $0.5 \times 10^8$  cells/ml) in lysis buffer (50mM Tris, 150mM NaCl, 2mM DTT, 1% Triton-X-100, pH 7.8) by vortexing and then lysed by dounce homogenisation (10 strokes). Lysates were incubated in the cold room on a roller for 2 hours. The lysates were then centrifuged at 75,000g for 60 minutes and the supernatant was collected.

*GST affinity purification:* GST purifications were performed using glutathione sepharose 4B beads (GE healthcare). 1ml of settled beads were equilibrated in lysis buffer and then incubated with the lysates in the cold room on a roller for 30 minutes. The beads were then collected using a plastic drip column. The collected beads were washed with 20 column volume wash buffer (50mM Tris, 150mM NaCl, 2mM DTT, 0.05% Triton-X-100, pH 7.8) and then 0.5ml elution fractions were collected using the elution buffer (50mM Tris, 150mM

NaCl, 2mM DTT, 0.05% Triton-X-100, 50mM glutathione, pH 7.8). The elutions obtained were analysed by Coomassie stained SDS-PAGE and typically elutions 2-5 were stored at -80°C and used for the gel filtration chromatography.

*Gel filtration purification:* The purification buffer used for the gel filtration chromatography was 50mM Tris, 150mM NaCl, 2mM DTT, 0.05% Triton-X-100, pH 7.8. Gel filtration purifications were performed using an Akta prime (GE healthcare) at a flow rate of 1ml/minute using the HiLoad 16/600 Superdex 200 pg column (GE healthcare). The column was equilibrated in 1 column volume of purification buffer after which 2ml (~2mg) of the GST elutions was loaded and 1ml fractions were collected. Fractions corresponding to individual peaks in the chromatogram (A280) were pooled and analysed by Coomassie stained SDS-PAGE (20µl sample). A chromatogram (provided by the manufacturer) of protein standards separated using this column was used to determine the approximate molecular weights of the fractions obtained following the gel filtration purification. Purity was estimated using Coomassie stained gels and the ImageJ software by calculating the percentage of the total peak sizes attributed to the band corresponding to GST-DAGLβ CD.

## Western blotting

Samples were diluted using water and 5x SDS protein loading buffer to a concentration of 1µg/µl and then denatured by boiling for 5 minutes. 10µg of the denatured samples were resolved by SDS-PAGE (4% stacking and 10% resolving tris-glycine gels) at a setting of 100 volts for ~2 hours. The resolved proteins on the gel were transferred (wet transfer) to a nitrocellulose membrane (GE healthcare) for 1 hour at 100 volts (4°C). Following the transfer, membranes were blocked for 1 hour at room temperature in PBS 5% milk and then incubated overnight at 4°C with the primary antibody (V5 (mouse; Invitrogen) or GST

(rabbit; Millipore) antibody) diluted 1/1000 in PBS 0.1% Tween (PBST) 2% milk. The membranes were then washed in PBST (three times for 10 minutes) and incubated for 1 hour at room temperature with the corresponding mouse IR-Dye 680 or rabbit IR-Dye 800 secondary antibody (LI-COR, diluted 1/5000 in PBST 2% milk). Following this, the membranes were washed in PBST (4 times, 10 minutes each) and then PBS (1 time for 10 minutes). The membranes were visualised using the Odyssey imaging system (LI-COR).  $\beta$ -actin (rabbit antibody, Abcam) was also detected as a loading control for the cell membranes.

**Assays**

96-well clear polypropylene plates were used for the assays. The PNPB and DiFMUO (Invitrogen) membrane assays were performed as previously described with some modifications<sup>33</sup>. The assay buffer for the PNPB membrane assay was 50mM HEPES pH 7.5, 5% DMSO and for the DiFMUO assay - 50mM MES pH 6.5, 5% DMSO. Membranes were typically diluted to 4x the final assay concentration (FAC) using the assay buffer, and then 50 $\mu$ l/well was dispensed. 50 $\mu$ l of the drug (4x FAC, diluted using assay buffer) or assay buffer was then added to the membranes followed by 5 minute incubation at room temperature. The substrate (DiFMUO or PNPB) was diluted to 40x FAC using DMSO and then to 2x FAC using the assay buffer without DMSO. 100 $\mu$ l of the diluted substrate was added to each well and the plates were read. For the PNPB assay, the OD400 was measured every 12 seconds for 30 minutes using a Spectramax plate reader (Molecular Devices). For the DiFMUO assay, the fluorescence (excitation 360nm, emission 450nm) was measured every 30 seconds for 30 minutes using the FlexStation (Molecular Devices). The total assay volume was 200 $\mu$ l containing 12.5 $\mu$ g/ml membranes, 250 $\mu$ M PNPB or 10 $\mu$ M DiFMUO and

5% DMSO. The reaction rates were calculated over the first 10 minutes (linear) using 3 replicate wells.

For the EnzChek (Invitrogen) assay, membranes or GST-DAGL $\beta$  CD were typically diluted to 4x FAC using 50mM HEPES pH 7.5, 5% DMSO (assay buffer) and then 50 $\mu$ l was dispensed in each well. 50 $\mu$ l of the drug (4x FAC) or assay buffer was added to the wells and the plate was then incubated for 5 minutes at room temperature. The substrate EnzChek was diluted to 40x FAC using DMSO and then to 2x FAC using the assay buffer without DMSO. 100 $\mu$ l of the diluted substrate was added to each well and the fluorescence (excitation 480nm, emission 540nm) was measured every 48 seconds for 10 minutes using the FlexStation. The total assay volume was 200 $\mu$ l, typically containing 12.5 $\mu$ g/ml membranes or specified amount of GST-DAGL $\beta$  CD, 2 $\mu$ M EnzChek, and 5% DMSO. The reactions rates were typically calculated over the first 2 minutes (linear) using 3 replicate wells.  $K_m$  and  $V_{max}$  were determined using the SigmaPlot v13 software (Systat Software Inc.) and Michaelis–Menten equation.

**Results**

**DAGLβ activity membrane assay**

To support the development of a DAGLβ assay, a U2OS cell line stably expressing DAGLβ-V5 was established following verification by both Western and immunocytochemistry analyses using a V5 antibody (Figure 1A and B). Stable expression of DAGLβ-V5 was confirmed beyond 30 passages in this cell line (V5β4). We measured the activity of membranes derived from V5β4 as well as parental controls using the previously reported DAGLα surrogate substrates PNPB and DiFMUO<sup>33</sup>. Following optimisation of various parameters including assay buffer constituents and the substrate and membrane concentrations a relatively modest but significant 2-fold difference in the tetrahydrolipstatin (THL) sensitive activity was detected between the V5β4 and parental membranes when using PNPB as the substrate (Figure 1C) whereas no significant difference in activity was observed when using DiFMUO as the substrate (Figure 1D). This was in stark contrast to results obtained using membranes derived from the U2OS cell line stably expressing DAGLα-V5 (V5α11) which were used as controls to validate the assay conditions and were found to be significantly more active than the parental membranes in both the PNPB (10-fold) and DiFMUO (2-fold) assays when compared to the V5β4 membranes (Figure S1A and B). These results indicate that PNPB and DiFMUO are relatively poor substrates of DAGLβ. To investigate this further and in an effort to establish a more robust DAGLβ activity assay, we tested the activity of DAGLβ against a third commercially available substrate, EnzChek. An improved 3.4 fold difference in activity was observed in the V5β4 compared to the parental cell membranes indicating that EnzChek is a more favourable substrate of DAGLβ compared to PNPB or DiFMUO. Intriguingly, unlike the PNPB and DiFMUO assays, the V5α11

membranes were less active than the V5β4 membranes when using EnzChek as the substrate (Figure S1C).

### Development of a DAGLβ EnzChek assay using the purified catalytic domain

In order to generate a relatively clean DAGLβ assay with minimal background activity and a homogenous enzyme source, we expressed and purified the N-terminally tagged GST catalytic domain of DAGLβ using the baculovirus expression system and GST affinity and gel filtration chromatography. Firstly, we conducted an expression time course using recombinant baculoviruses containing the GST-DAGLβ CD transgene and Sf9 insect cells. 48 hours was selected as the harvest time for scale-up batches based on expression levels detected in lysates at the predicted molecular weight (~75kDa) by Western analyses using a GST antibody as well as levels of lower molecular weight GST tagged contaminants which could prove problematic during purification (Figure 2A).

Following scaled up expression and GST affinity purification, the elutions were analysed by Coomassie stained SDS-PAGE. A prominent band at the predicted size of GST-DAGLβ CD was detected in the elutions (Figure 2B). The purity was adjudged to be ~25% with the major contaminants detected at ~25kDa. Due to the significant size difference between the major contaminants and the GST-DAGLβ CD, gel filtration chromatography was used to further purify the enzyme using the HiLoad 16/600 Superdex 200 column. 2ml of the GST elutions were subjected to gel filtration chromatography. Any fractions corresponding to peaks in the chromatogram (absorbance at 280nm), were analysed by Coomassie stained SDS-PAGE (Figure 2C; the fraction numbers correspond to the elution volume). The chromatogram was also used to estimate the native molecular weights of the fractions. The activity of the fractions was also tested using EnzChek (Figure 2D). The first three pooled fractions



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contained GST-DAGLβ CD; the first (44-49) and second (50-60) corresponded to a native molecular weight >400kDa and were found to be largely inactive thereby indicating that the GST-DAGLβ CD present in these samples was aggregated or misfolded. The third pooled fraction (62-68) corresponded to a native molecular weight of ~160kDa and was the most active indicating that the sample contained active dimeric GST-DAGLβ CD; the activity was largely inhibited by THL (~10-fold THL sensitive window). The subsequent pooled elutions (69-74; 76-80; 81-90) contained lower molecular weight contaminants and/or little or no GST-DAGLβ CD/THL sensitive activity. Fraction 62-68 was therefore aliquoted and stored at -80°C and used for subsequent experiments. The yield following gel filtration chromatography was 220μg/L of culture volume and the estimated purity of the sample was ~90%.

In order to optimise a GST-DAGLβ CD EnzChek assay, we first monitored activity rates as a function of enzyme concentration. Increasing amounts of enzyme corresponded to increasing rates of activity and the signal was found to be linear for the duration of the assay (10 minutes) (Figure 3A and B). Almost no activity was detected in the absence of GST-DAGLβ CD. For further experiments 500ng of enzyme was used per well. We next determined the effect of substrate concentration on the rate of activity (Figure 3C). Increasing concentrations of EnzChek was associated with increasing rates of activity. The Km and Vmax were determined as 4.26±0.32μM and 4.95±0.15 RFU/sec respectively.

In order to further validate the assay, two established DAGL inhibitors (THL and OMDM-188) were tested at increasing concentrations. Both compounds inhibited activity in a concentration dependent manner with IC50s determined as follows – THL 26.86±4.33nM (Hill slope 0.95±0.13) and OMDM-188 6.70±2.05nM (Hill slope 0.79±0.17) (Figure 4). The results are consistent with previous DAGLα studies where OMDM-188 was reported as the

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3 more potent DAGL inhibitor <sup>33,35,36</sup>. The intra and inter-assay coefficient of variations were  
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5 determined as 4.5% (n=4) and 9.8% (N=5) respectively.  
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Discussion

The DAGLs are emerging targets for a range of diseases and are therefore of great interest for drug discovery programmes. Native substrate DAGL assays involve the use of synthetic DAG as a substrate coupled with the measurement of 2-AG as the product and generally rely on low-throughput mass spectrometry techniques <sup>26,27</sup>. Other low-throughput thin layered chromatography based assays using radio-labelled substrates <sup>2,28</sup> and gel based assays using activity based probes <sup>13,27,29,30</sup> have also been reported. There have been recent reports of DAGL activity assays that are amenable to relatively high-throughput screening programmes for inhibitors or activators of these enzymes; the assays reported generally employ membranes or lysates isolated from cells over-expressing DAGL $\alpha$  <sup>28,33</sup> and notably include a DAGL $\alpha$  native substrate assay using a coupled enzyme approach adapted from a previously reported MAGL assay <sup>31,32</sup>.

In order to further complement the panel of DAGL activity assays, we report here human DAGL $\beta$  activity assays using membranes over-expressing the full length enzyme as well as its purified catalytic domain. By establishing the U2OS cell line (V5 $\beta$ 4) that stably and robustly express the human DAGL $\beta$  transgene, we have developed a consistent tool (compared to transiently transfected cells) to directly study human DAGL $\beta$  catalytic activity and attempted to adapt previously reported DAGL $\alpha$  substrate assays for this purpose <sup>33</sup>. The difference in activity between the V5 $\beta$ 4 and parental membranes was higher when using the smaller chromogenic PNPB substrate (2 fold) in comparison to the larger fluorogenic substrate DiFMUO (no significant difference). Possible explanations for this difference could be higher background enzymatic activity in the parental membranes towards DiFMUO when compared to PNPB which could especially be problematic as DiFMUO may access the DAGLs through the membrane whereas PNPB may access the DAGLs through the aqueous phase. If this is the case, then the membrane diffusion rates of DiFMUO could prove to be

rate limiting in the context of DAGL activity<sup>33</sup>. If both substrates were to access the DAGLs through the membrane, then different membrane diffusion rates could explain the differences in activity. The higher molecular weights of DiFMUO (338Da) when compared to PNPB (209Da) may also influence the activity of the DAGLs against these substrates as substrate access to the active site of lipases is known to be regulated by a displaceable lid like structure<sup>37–39</sup>. The smaller PNPB substrate could potentially be less susceptible to steric hindrance by the lid when compared to larger substrates. On the contrary, however, the DAGL $\beta$  membrane assay employing the larger fluorogenic EnzChek substrate (1011Da) was found to be superior to the PNPB and DiFMUO assays based on the difference in activity between V5 $\beta$ 4 and parental membranes (3.5 fold using EnzChek compared to 2 fold using PNPB and no significant difference using DiFMUO). As part of the study, membranes derived from the cell line stably expressing DAGL $\alpha$ -V5 were employed as controls for the PNPB and DiFMUO assays and were found to be much more active than the V5 $\beta$ 4 membranes. Establishing relative expression levels of DAGL $\alpha$  and DAGL $\beta$  in the membranes will help determine whether these differences in activity reflect quantitative and/or qualitative differences between these two enzyme preparations. Intriguingly, in contrast to the PNPB and DiFMUO assays, the V5 $\alpha$ 11 membranes were less active compared to the V5 $\beta$ 4 membranes in the EnzChek assay which thereby indicates differences in the catalytic activity of these closely related enzymes. More detailed kinetic studies involving the DAGLs and these substrates are warranted in order to help elucidate structural details that may contribute to these differences in activity, especially as such details could be of potential interest from a medicinal chemistry perspective.

Activity assays involving purified enzymes provide homogenous, consistent, and reliable platforms for high-throughput screens by minimizing background and/or non-specific activity often observed in crude samples. We successfully expressed and purified the N-terminally

GST-tagged catalytic domain of DAGL $\beta$  (GST-DAGL $\beta$  CD) using the baculovirus expression system and GST affinity and gel filtration chromatography achieving yields of ~0.2 mg/L culture volume and purity of ~90%. As in our case, the baculovirus expression system has proven to be a valuable tool for the expression and purification of a range of challenging mammalian targets that are too complex and prone to aggregation in the simpler prokaryotic *E. coli* expression system<sup>40–42</sup>. Activity of GST-DAGL $\beta$  CD was confirmed using EnzChek (which was the best substrate identified here using DAGL $\beta$  membranes) as well as two established DAGL $\beta$  inhibitors, namely THL, and OMDM-188<sup>2,27,33</sup>. As far as we are aware, this is the first report of the purification of recombinant mammalian DAGL which besides its obvious utility in drug discovery assays may also prove valuable for other studies. For example, in the absence of crystal structures for the DAGLs, mutagenesis studies coupled with activity assays using the substrates described here and in other reports could help identify structural features of this enzyme. Moreover, insertion of a cleavage site between the GST tag and catalytic domain could enable the purification of the untagged catalytic domain for structural studies using X-ray crystallography or other techniques. Additionally, *in vitro* phosphorylation coupled with mutagenesis studies may help elucidate activation mechanisms considering that the lid of DAGL $\beta$  contains a hyper-phosphorylated region<sup>39</sup>.

Future work comparing GST-DAGL $\beta$  CD to full length recombinant and endogenous DAGL $\beta$  will be important to establish further utility for this purified enzyme. For example, characterizing GST-DAGL $\beta$  CD using the native DAGL substrate 1-stearoyl-2-arachidonoyl-sn-glycerol will enable comparisons with previously reported  $K_m$  values for recombinant full length DAGL $\beta$  ( $K_m$  74.1 $\pm$ 4.9 $\mu$ M using using sn-1-stearoyl-2-[14C]arachidonoyl-glycerol as the substrate)<sup>2</sup>. Similarly, immunoprecipitation, co-immunoprecipitation, and native SDS-PAGE studies to determine the oligomeric (or monomeric) status of full length endogenous and/or recombinant DAGL $\beta$  will be useful as a preliminary quaternary structure comparison

with the purified dimeric GST-DAGL $\beta$  CD. As far as the activity assay is concerned, including product standards within the assay plate will enable the quantification of the product and thereby comparisons with activities measured using other DAGL substrates (e.g. the native substrate 1-stearoyl-2-arachidonoyl-sn-glycerol). Determining the Z' factor of the assay will also be important towards validating the assay for high-throughput screening campaigns<sup>43</sup>. It is noteworthy to mention that some serine protease inhibitors like PMSF which are commonly used as part of protein purification procedures can also potentially inhibit serine hydrolases like the DAGLs and therefore, as part of future efforts, screening various protease inhibitors will be advisable to improve the assays performance. Characterizing further established (e.g. RHC-80267) as well as recently discovered novel DAGL inhibitors in this “clean” and homogenous assay will not only further validate the assay but also potentially provide useful information regarding their inhibition kinetics and enable comparisons with their DAGL $\alpha$  inhibition profiles<sup>2,13,34,44,45</sup>. In general, the DAGL $\beta$  assays described here also have particular utility as selectivity assays. For instance, the DAGLs make distinct contributions towards 2-AG (and AA) levels in different tissues e.g. DAGL $\alpha$  accounts for most of the 2-AG in the brain, DAGL $\beta$  is responsible for most of the 2-AG in the liver<sup>7</sup>. The use of selective DAGL $\beta$  inhibitors to inhibit eCB signalling might be fruitful in certain conditions as it has the potential to avoid the adverse psychiatric effects of blocking the pathway at the level of the CB1 receptor<sup>25</sup> as it is DAGL $\alpha$  rather than DAGL $\beta$ , that is responsible for generating 2-AG at synapses<sup>7</sup>. Similarly, the selective expression of DAGL $\beta$  in macrophages and microglia makes this enzyme a very interesting therapeutic target for inflammatory diseases in both the periphery and central nervous system, and importantly this operates independently from the cannabinoid receptors<sup>13,23,24</sup>. Targeting DAGL $\alpha$  or DAGL $\beta$  selectively could therefore be therapeutically attractive, and a further understanding of structural and functional differences between these enzymes will be pivotal

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towards achieving this. Encouragingly, the recent development of a DAGL $\beta$  selective inhibitor (50 times more potent against DAGL $\beta$  than DAGL $\alpha$ ) has demonstrated that this is indeed achievable from a medicinal chemistry perspective <sup>13</sup>.

In conclusion, the relatively simple DAGL $\beta$  assays reported here utilizing recombinant membranes as well as its purified catalytic domain have great utility for drug discovery programmes as a primary (and selectivity) assay with the potential to be adapted for structural and mechanistic studies to help further our understanding of this emerging and promising anti-inflammatory target.

## Acknowledgments

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## Supporting Information Available

Figure S1 - Characterising DAGL $\alpha$  activity in the V5 $\alpha$ 11 membranes.

Supporting materials may be accessed free of charge online at <http://pubs.acs.org>.



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## Figure legends

### Figure 1. Characterising DAGL $\beta$ expression and activity in the V5 $\beta$ 4 membranes

A representative image of fixed V5 $\beta$ 4 cells immunostained with a V5 antibody (green) and the nuclear stain Hoechst (blue) is shown above, demonstrating the stable and clonal expression of DAGL $\beta$ -V5 (A). A representative Western blot image of membranes (10 $\mu$ g) isolated from V5 $\beta$ 4 cells is also shown, confirming DAGL $\beta$ -V5 expression at a size close to the predicted molecular weight (75kDa) (B). Parental cells/membranes were included as a negative control for both analyses. The activity of membranes (12.5 $\mu$ g/ml) isolated from the V5 $\beta$ 4 cells or parental cells was measured using PNPB (250 $\mu$ M) (mean $\pm$ SEM, V5 $\beta$ 4 n=9, parental n=13) (C) or DiFMUO (10 $\mu$ M) (mean $\pm$ SEM, V5 $\beta$ 4 n=4, parental n=8) (D) in the presence or absence of THL (1 $\mu$ M); reaction rates were calculated over the first 10 minutes and the THL sensitive activity is presented above. Activity of the membranes was also measured using a third substrate, EnzChek (2 $\mu$ M). The reaction rates were calculated over the first two minutes (mean $\pm$ SEM, n=3) and the THL sensitive activity detected is presented above (E). \*\* p<0.01, \* p<0.05 (two tailed t-test).

### Figure 2. Expression and purification of GST-DAGL $\beta$ CD

Lysates of Sf9 cells expressing GST-DAGL $\beta$  CD was analysed at different time points by Western blotting using a GST antibody which revealed 48 hours as the optimum time point (A). For the GST affinity purification, clarified lysates obtained from insect cells expressing GST-DAGL $\beta$  CD were incubated with glutathione sepharose 4B beads; bound protein was eluted using 50mM glutathione and elutions were analysed by Coomassie stained SDS-PAGE (B). 2mls of the elutions from the GST affinity purification were loaded onto the HiLoad



16/600 Superdex 200 column (gel filtration purification). Fractions corresponding to peaks observed in the chromatogram (absorbance at 280nm) were collected and the corresponding Coomassie stained SDS-PAGE analysis (20µl) of the fractions is presented above (C). The first three sets of pooled fractions contained GST-DAGLβ CD. Activity of these fractions was tested using EnzChek (2µM) in the presence or absence of THL (1µM); the reaction rates were calculated over 10 minutes and are presented above (mean of 3 wells ±SEM). THL sensitive activity was largely detected in fractions 62-68 (D).

**Figure 3. Development of a GST-DAGLβ CD activity assay**

The activity of different amounts of GST-DAGLβ CD following GST affinity and gel filtration chromatography was measured using EnzChek (2µM) by measuring the fluorescence (excitation 480nm, emission 540nm) every 48 seconds for 10 minutes. Increasing amounts of enzyme corresponded to increasing rates of activity measured over 10 minutes (mean of 3 wells ±SEM) (A) during which the reaction was found to be linear (B). The activity of 500ng GST-DAGLβ CD was tested with different concentrations of EnzChek as mentioned above (mean of 3 wells ±SEM) (C). Km was determined as 4.89±0.66µM.

**Figure 4. Inhibition of GST-DAGLβ CD activity**

The activity of GST-DAGLβ CD (500ng) following GST affinity and gel filtration chromatography was measured using EnzChek (2µM) by measuring the fluorescence (excitation 480nm, emission 540nm) every 48 seconds for 10 minutes in the presence of two DAGL inhibitors. Both compounds, namely THL and OMDM-188 (mean±SD, n=2) were found to inhibit activity.

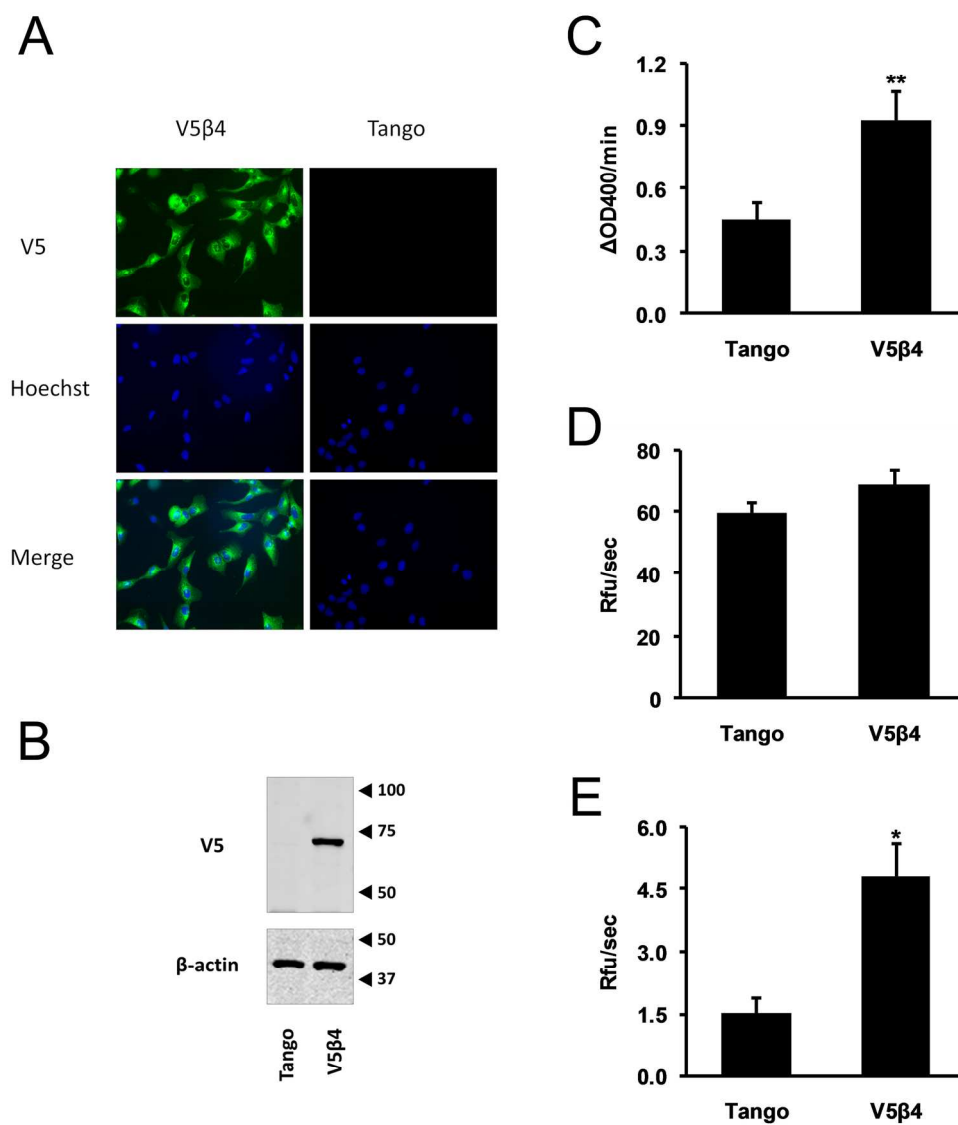


Figure 1. Characterising DAGLβ expression and activity in the V5β4 membranes  
210x250mm (300 x 300 DPI)

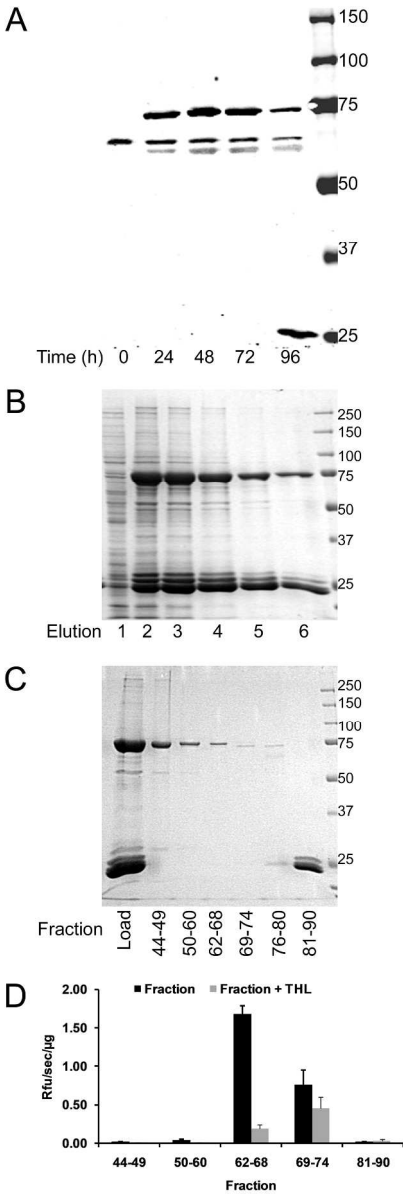


Figure 2. Expression and purification of GST-DAGLβ CD  
228x616mm (300 x 300 DPI)

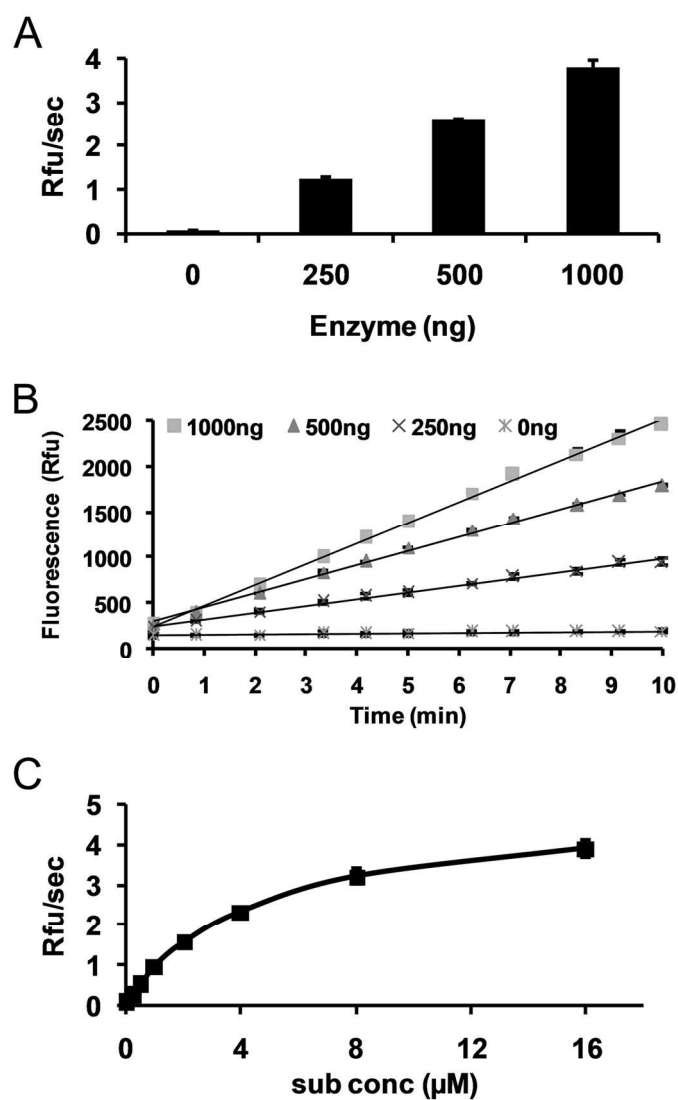


Figure 3. Development of a GST-DAGL $\beta$  CD activity assay  
131x204mm (300 x 300 DPI)

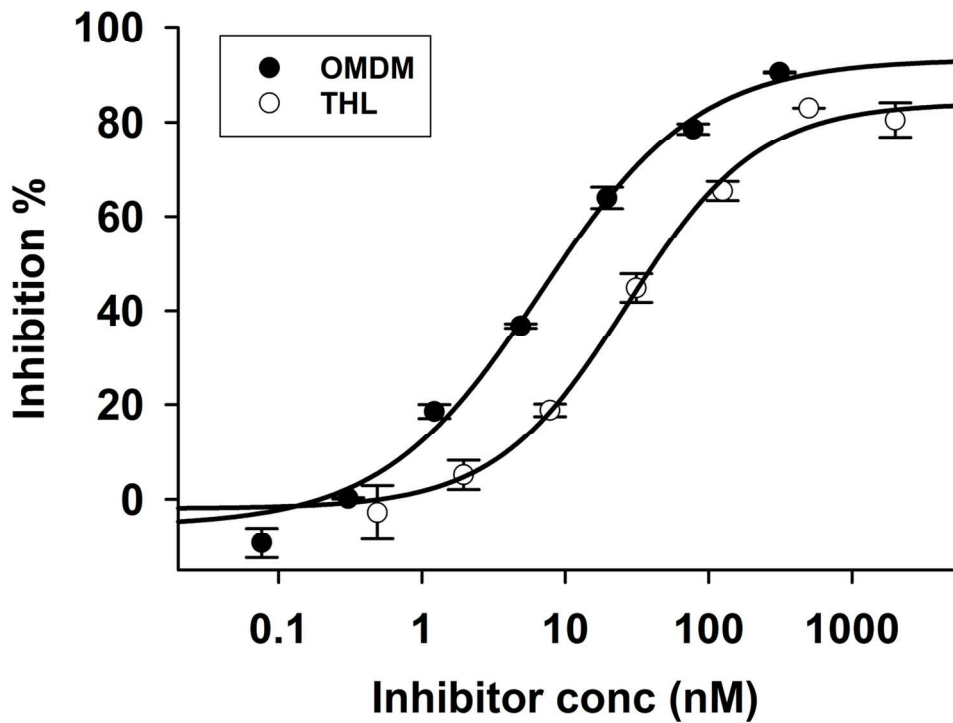
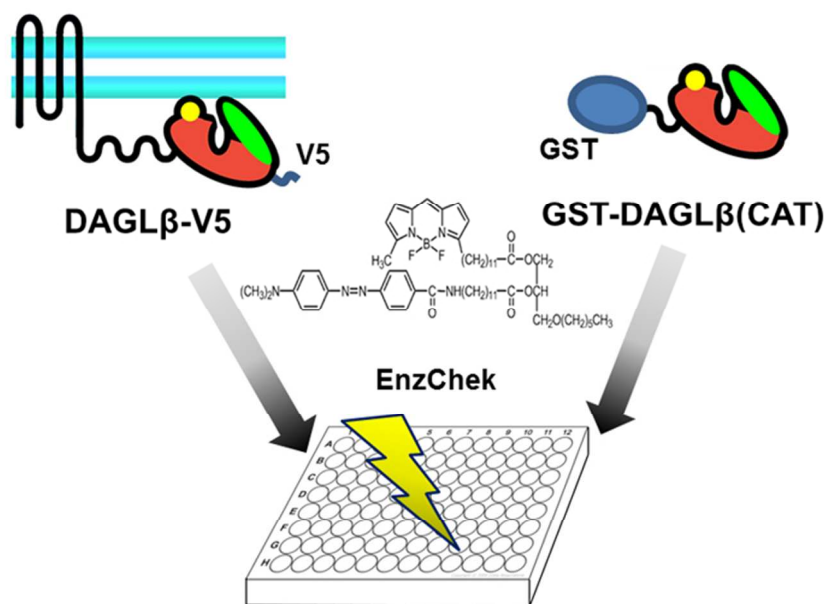


Figure 4. Inhibition of GST-DAGLβ CD activity  
63x50mm (600 x 600 DPI)



Graphic image for use within Table of Contents only  
70x44mm (300 x 300 DPI)